

The University of Chicago

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CHYLE

A DISSERTATION SUBMITTED TO THE
FACULTY OF THE DIVISION OF THE
BIOLOGICAL SCIENCES IN CANDIDACY FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF PHYSIOLOGY

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THE HEMOLYTIC ACTION OF CHYLE¹

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It has been previously demonstrated that samples of lymph collected from the lacteals and thoracic ducts of dogs from 3 to 5 hours or more after the ingestion of fat are strongly hemolytic (Johnson and Freeman, 1938), by making ordinary red blood cell counts on mixtures of equal quantities *a*, of whole blood plus lymph² (about 0.015 cc. of each), and *b*, of blood plus Ringer's solution. Almost without exception the red blood cell counts made on blood and chyle mixtures were lower than the counts made on blood and Ringer's solution mixtures. The supernatant fluid of centrifuged samples of blood and chyle mixtures also showed definite evidence of hemolysis. These findings have been confirmed repeatedly, using the same technique with slight modifications.

What is this factor in chyle which causes hemolysis? The substance is found in the lacteals and thoracic duct only after fat-feeding. Lymph obtained from these sources in fasting animals or from cervical or foot lymphatics in fat-fed animals does not hemolyze red blood cells when mixed in equal quantities with whole blood. Thus, it is a substance which appears in the lymph only during the transport of products of fat absorption. The following substances were considered:

1. *Cholesterol*. This has been shown repeatedly to be antagonistic to the hemolytic action of such substances as fatty acids and saponin (Meyerstein, 1912; Brinkman, 1929). Therefore, it was not considered to be an important causative factor.

2. *Neutral fat*. This is probably not an important factor since mixtures of equal quantities of blood plus milk or cream give the same red blood cell counts as blood mixed with Ringer's solution.

3. *Bile salts*. Sodium taurocholate is definitely hemolytic and might be involved in the hemolytic action of chyle. Since good quantitative methods for bile salt determination are lacking, no chemical estimations were

¹ This work was in part aided by a grant from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

² Heparin was used as an anti-coagulant for both blood and lymph in some cases. In other instances, intravenous injection of Chlorazol Fast Pink BKS (Modell, 1939) prevented coagulation of both blood and lymph.

attempted. However, the introduction of bile or bile salts into the intestine yielded lymph possessing little or no hemolytic activity. Therefore, it seems that the rôle of bile salts in the hemolytic activity of chyle is probably insignificant.

4. *Osmotic pressure.* No direct measurements of the osmotic pressure of chyle were made in this study, but the work of several investigators (summarized by Drinker and Field, 1933) indicates that the total osmotic pressure of lymph is even slightly higher than that of serum. Furthermore, many mixtures of potently hemolytic chyle and whole blood showed crenation of the red blood cells. These findings suggest that salt osmotic pressure changes are not involved in the hemolytic action.

5. *Hydrogen ion concentration.* Determinations were made of hemolytic potency³ and the pH of lymph samples collected under oil. The pH was determined by means of a glass electrode and was found to remain almost constant throughout an experiment, having no apparent relationship to hemolytic potency. Table 1 is a typical protocol.

TABLE 1

The hemolytic potency and pH of thoracic duct lymph collected after fat feeding

TIME AFTER FAT FEEDING	HEMOLYTIC POTENCY OF LYMPH	pH OF LYMPH
<i>hours</i>	<i>per cent</i>	
3 $\frac{2}{3}$	20	7.357
4	34	7.357
4 $\frac{1}{2}$	6	7.341
4 $\frac{3}{4}$	3	7.349

6. *Temperature.* The temperature of the mixtures was kept constant without varying the results.

7. *Enzymes.* Chyle samples were collected and divided into two portions. One portion was heated to 75°C. for 10 minutes; the other was untreated. No constant or significant differences in hemolytic potency of heated and unheated samples were observed.

8. *Glycerol.* In an earlier report (Johnson and Freeman, 1938) it was suggested that glycerol might be responsible for the hemolytic action of chyle. Other investigators (Ramond and Flandrin, 1904) had demonstrated large amounts of glycerol in the lacteals and thoracic ducts of dogs,

³ Throughout the paper, percent hemolysis is used as an expression of hemolytic potency of samples tested. It is determined by the formula $\frac{C_R - C_L}{C_R} \times 100$, where C_R is the control red blood cell count made upon mixtures of equal quantities of whole blood plus Ringer's solution and C_L is the red blood cell count made upon mixtures of equal quantities of whole blood plus the sample (usually lymph) whose hemolytic potency is being determined.

but they reported concentrations equally as high in the portal and peripheral blood streams, where presumably no hemolysis occurs. In the present study, glycerol was placed directly into washed, intact intestinal loops, or glycerol was fed with diets not containing fat in a number of dogs. This yielded thoracic duct lymph which was not hemolytic. These considerations seem to indicate that glycerol is probably not the substance which causes hemolysis.

9. *Free fatty acids and soaps.* Of the substances found in the gut during fat digestion, there remain free fatty acids and soaps. Might not quantities of these sufficient to cause hemolysis escape resynthesis into neutral fat and be absorbed into the lacteals? Several lines of evidence supported this hypothesis. Placing oleic acid or sodium oleate in washed intestinal loops of dogs gave intensely hemolytic chyle. Also, earlier workers (Faust and Tallqvist, 1907) fed fatty acid to one dog and a cholesterol ester to another, getting lymph with hemolytic power. They extracted the lymph with fat solvents, made a suspension of the dissolved material and showed that this also was hemolytic.

In an attempt to discover whether free fatty acid and soap were responsible for the hemolytic action of chyle, quantitative analyses for soap and free fatty acid were made. The analytic method employed combined features of methods published by several workers (Boyd, 1936; Bloor, 1915; Fowweather, 1926; Stoddard and Drury, 1929). Briefly, the method was as follows:

A 3:1 alcohol-ether mixture (both freshly redistilled) was acidified with 1 cc. of 3 per cent hydrochloric acid for each 50 cc. One cubic centimeter of the fluid containing fatty acid and soap was run slowly into 35 cc. of the mixture. This was heated to about 60°C. for two minutes, stirred to prevent superheating, cooled and centrifuged. The supernatant fluid was then poured into a 50 cc. volumetric flask and the residue was re-extracted with 15 cc. of the alcohol-ether. Two 20 cc. aliquots of the alcohol-ether extracts were placed in evaporating dishes containing extracted and washed sand and were evaporated to dryness with gentle heat on a steam bath. The dry residues were each dissolved in 25 cc. of petroleum ether and were filtered through fat-free filter paper. The duplicate samples of petroleum ether extract were heated almost to boiling and were titrated with approximately 0.02 normal sodium ethylate to a pink that did not change, using phenolphthalein as the indicator. Blanks, checks and knowns were run through the whole procedure.

Checks on the method consisted in adding known quantities of soap and fatty acid to lymph or Ringer's solution, and then analysing these solutions and emulsions as described above. The results are shown in figure 1, in which known milligrams added are plotted against milligrams detected by analysis. If the recovery were perfect, all the points would

fall on the straight line drawn into the figure. The distribution of the points shows that not quite all of the added soap or acid was recovered. However, the results were considered sufficiently accurate for the purposes of this study.

Next, the extent of hemolysis produced by mixing red blood cells with Ringer's solutions to which known amounts of fatty acid and soap were added *in vitro* was determined. These findings are recorded graphically in figure 2, in which the milligrams of added soap or fatty acid are plotted against the hemolytic potencies. Lines are drawn connecting determinations made on individual animals. Although the fluctuations from animal to animal are considerable, it is apparent that there is a positive correlation between quantity of fatty acid or soap added and the hemolytic potency of the solution or emulsion.

Finally, samples of thoracic duct lymph were analysed for soap and free fatty acid content. In fasting dogs, whose lymph showed no hemolytic activity, the quantities of soap and free fatty acid ranged from 1.0 to 2.0 mgm. per cubic centimeter—too little to produce detectable hemolysis. Values ranging from 3.3 to 6.3 mgm. per cubic centimeter were found in the course of $3\frac{1}{2}$ to $4\frac{1}{2}$ hours following fat-feeding. In a few instances, soap and free fatty acid were determined separately on each sample. The concentration of free fatty acid alone in chyle rarely reached 1 mgm. per cubic centimeter. Using somewhat different methods, other investigators have also found free fatty acids and soaps in chyle. Faust and Tallqvist (1907) found about 12 mgm. of soap plus free fatty acid in the thoracic duct lymph of a dog fed an oleic acid-cholesterol ester, and even more in a dog fed oleic acid. Munk (1880) also presents larger values for free fatty acid and soap than were found in the present study. Hoppe-Seyler (1879) calculated that from 2.5 to 4.0 mgm. of soap enter the blood stream by way of the chyle every minute following the ingestion of fat. The work of Freeman and Friedemann (1935) indicates that about 12 per cent of the fatty acids in chyle are uncombined. Lastly, Artom and Peretti (1935) found that 2 per cent of the fatty acids were present in chyle as soaps and free fatty acids.

Are the values reported here sufficient to account for the hemolysis observed? In each sample analysed chemically, the hemolytic potency was also determined. In figure 3 the results are plotted, showing the relationship of the quantity of soap or fatty acid detected in lymph to the hemolytic potency of the lymph. The line drawn in figure 3 is derived from the data shown in figure 2 by connecting the averages of the points on figure 2. Points in figure 3 lying above the line represent lymph samples containing at least enough soap or fatty acid to account for the extent of hemolytic potency observed. That the line in figure 3 is not placed too low is borne out by observations of Edwards (1939), McPhedran (1913), and Zinck, Clark and Evans (1922) who were able to produce definite

hemolysis with solutions of fatty acids and soaps even less concentrated than the solutions used in the present study.

The data in figure 3 seem to indicate that, in general, the quantity of soap and fatty acid found in chyle is adequate to account for the hemolytic potency. One can only speculate on the failure of the points to distribute themselves along a straight line. Perhaps the variations in the resistance of corpuscles from different animals to these agents are partly responsible. The data of figure 2 support this view. Perhaps there are variations in the anti-hemolytic action of serum, or in the concentrations of cholesterol, an anti-hemolytic agent. Several workers (Liebermann, 1907; Noguchi, 1907; Meyer, 1908; Meyerstein, 1912; Zinck, Clark, and Evans, 1922;

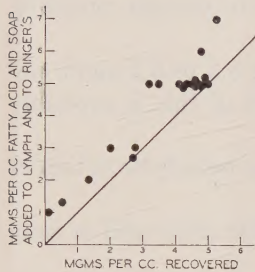


Fig. 1

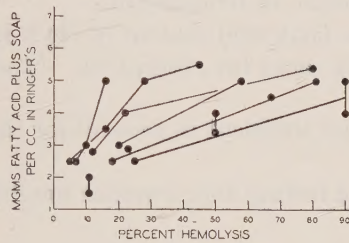


Fig. 2

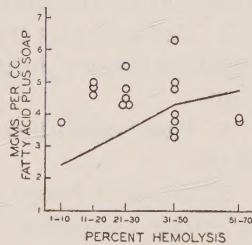


Fig. 3

Fig. 1. Relation of added milligrams of soap and free fatty acid to milligrams as detected by analysis. The straight line indicates the theoretical location of the points if the recoveries were complete.

Fig. 2. Relation of amount of soap or fatty acid added to Ringer's solution (in vitro) to extent of hemolysis observed when this was mixed with equal quantities of whole blood. Lines are drawn through determinations made on the cells of individual animals.

Fig. 3. Relation of hemolytic potency to quantity of fatty acid and soap determined by chemical analysis. The line drawn represents the averages of points plotted in figure 2.

Brinkman, 1929) have shown that serum exerts a protective action against hemolysis by soaps and fatty acids. The presence of some anti-hemolytic activity is suggested by the distribution of the points to the left, in figure 3, since these points represent chyle samples containing more than enough fatty acid or soap to account for the hemolytic potency. Finally, there may be variable quantities of insoluble calcium soaps formed (Brinkman and Szent-Gyorgyi, 1923).

Whether or not the figures reported are of significance, physiologically or pathologically, remains to be determined. It has been suggested (Johnson and Freeman, 1938) that absorption of these hemolytic agents into the lymphatics may be of adaptive value, since instead of entering capillaries at once, where red cells might be damaged, the hemolysins enter the blood stream only after they have been diluted by lymph from

many parts of the body. Further, the diluted hemolysins are poured into a stream of blood returning from many regions, instead of initially entering and mixing with the blood of the intestine alone. It remains to be demonstrated that the direct entrance of these substances into the capillaries of the portal circulation would destroy or damage red blood cells.

SUMMARY AND CONCLUSIONS

1. The existence of a hemolytic agent in thoracic duct lymph, during absorption of ingested fat, is amply confirmed. Other lymph is not hemolytic.

2. Evidence is presented that this hemolytic agent is not cholesterol, neutral fat, bile salts, enzymes, glycerol, or changes in osmotic pressure, hydrogen ion concentration, or temperature.

3. The soap plus free fatty acid content of chyle is 3.3 to 6.3 mgm. per cubic centimeter during rapid fat absorption. Most of this is probably soap.

4. These quantities are sufficient to account for the hemolytic action of chyle.

5. The duct lymph of fasting dogs contains too little fatty acid or soap to produce hemolysis.

6. The possible significance of these findings is discussed.

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